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Adenovirus-mediated gene transfer: influence of transgene, mouse strain and type of immune response on persistence of transgene expression

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E1-deleted adenovirus (Ad) vectors expressing the human coagulation factor IX (hFIX) or the bacterial β-galactosidase (lacZ) were injected intravenously into various strains of immunocompetent (C57Bl/6, BALB/c, CD1, CBA/J, C3H) and immunodeficient (BALB/c-nu/nu, C57Bl/6-nu/nu, SCID, NIH-bg-nu-xid) mice. Regular analysis of mouse sera and tissues showed a persistent expression of both transgenes in immunodeficient mice, while detection diminished very rapidly in immunocompetent mice. The mechanisms responsible for the transient detection of the two transgenes were however not identical. Rapid decline of lacZ expression was correlated with a rapid decrease of viral DNA sequences, and consequently to the induction of a cellular immune response to the lacZ antigen. In contrast, absence of detectable levels of serum hFIX in immunocompetent animals was not associated with a loss of viral DNA but was strictly correlated with the induction of anti-hFIX antibodies. Surprisingly, anti-hFIX antibodies were never detected in C57Bl/6 mice, leading to prolonged detection of hFIX. These results suggest that cellular immunity to viral antigens plays a minor role in the early extinction of transgene expression and illustrate the influence of the cellular (eg lacZ) or humoral (eg hFIX) immunity to transgene-encoded products on the persistence of transgene expression.

Keywords: *adenovirus; factor IX; β-galactosidase; mouse; immune response*

Introduction

Human adenoviruses (Ad) are attractive candidate vectors for the direct *in vivo* transfer of potential therapeutic or vaccinal genes.[1–3] They are usually associated with benign pathologies in humans, the organization of their 36-kb double-stranded DNA genome is well established[4,5] and various methods for the generation of recombinant vectors are available.[6–10] Most current adenovirus vectors are derived from the Ad type 5 (Ad5) virus in which a foreign expression cassette has been introduced in place of the early region 1 (E1). Such E1-deleted vectors can be easily produced at high titers in complementation cell lines providing the E1 functions *in trans*.[11,12] The deletion of the regulatory E1 genes prevents viral propagation in infected organisms and dramatically reduces the expression of all viral genes.[1–3,13] Further deletion of the nonessential early region 3 (E3) genes allows insertion of larger segments of foreign DNA (up to 8 kb) in the viral genome. Such E1/E3-deleted vectors have already been used to transduce *in vivo* a wide range of both proliferating and quiescent cells with no, or limited, side-effects[14–22] and adenovirus-mediated gene transfer is currently considered for the genetic treatment of cystic fibrosis.[23–25] However, *in vivo* experimental evaluation of recombinant E1/E3-deleted adenoviruses has also revealed a major limitation to the use of such vectors in that expression of the transgene in the transduced tissues was found in most cases to be only transient.[14–16,18,21] These experiments suggested that the short-lived *in vivo* expression of the transgenes was most probably related to the induction of a host-specific cytotoxic T lymphocyte (CTL) immune response directed against viral antigens synthesized at low levels in the transduced tissues.[26–34]

Until very recently, the majority of published animal studies were, however, performed with adenovirus vectors expressing bacterial or human proteins. It is now clear that these proteins also constitute potential antigens recognized as foreign by the host immune system. In a recently published study, Tripathy *et al*[35] observed that administration to immunocompetent mice of vectors encoding the murine erythropoietin resulted in long-lasting elevated hematocrit levels. In contrast, injection of adenoviruses carrying the human erythropoietin gene induced a strong immune response directed against the human protein, which resulted in a transient expression of the transgene. The use of a broad range of transgenes from various origins may therefore strongly disturb the accurate determination of the specific immunological factors involved in the elimination of the transduced cells. Furthermore, any comparative evaluation of the immunogenicity of current E1/E3-deleted vectors with the supposedly less-immunogenic vectors deleted simultaneously in several viral regulatory genes[27,28,32,36–38] will similarly be disturbed by the presence of transgenes coding for immunogenic proteins. Also, most *in vivo*

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studies were done using inbred mouse strains of various haplotypes whose immune systems might react differently to a given antigen. Barr *et al*[39] reported that intravenous administration of an E1-deleted adenovirus vector carrying the human alpha-antitrypsin (hα-AT) cDNA leads to a strain-related variation in the persistence of expression of the transgene. The authors suggested that these variations were related to unidentified genetic loci that might influence the antigen-specific immune response. They did not however precisely determine the immunological factors responsible for the transient expression of hα-AT in C3H/HeJ and BALB/c mice and the longer persistence of expression in C57Bl/6 mice.

In this article, we report a similar strain-related variation of transgene expression in mice injected intravenously with a recombinant adenovirus carrying the human coagulation FIX (hFIX) cDNA and we describe the immunological component involved in the persistence of hFIX expression. We also investigated in nine different immunocompetent and immunodeficient mouse strains the influence of the mouse model, nature of transgene (bacterial β-galactosidase *versus* human FIX), type of immune response (humoral *versus* cellular) and targets of immunity (transgene-encoded products *versus* adenovirus antigens) on the *in vivo* persistence of the transduced cells.

Results

Persistence of hFIX and lacZ expression in immunocompetent mice

Recombinant AdhFIX and AdLacZ vectors (5×10^9 p.f.u.) were injected into the tail vein of inbred C57Bl/6, BALB/c, CBA/J, C3H and outbred CD1 mice. Sera and tissues (liver and lung) were then regularly collected and tested by ELISA for hFIX concentration (Figure 1a) and by histochemistry for *lacZ* expression (Figure 2c, d, g and h). In all animals (n = 23), hFIX values reached a peak level of 100 to 1000 ng/ml within 2 days of infection but rapidly decreased to undetectable levels in BALB/c, CBA/J, CD1 or C3H mice 1 month after virus injection (Figure 1a). The pattern of hFIX expression was surprisingly different in C57Bl/6 mice (n = 6). After a similar initial peak 2 days after inoculation, hFIX remained relatively persistent in the plasma during the course of the study. Despite a progressive decline, a concentration of 50 ng/ml of hFIX (around 5% of the peak value) was still detectable as late as 111 days after virus injection. A similar persistence of hFIX expression in C57Bl/6 mice only has already been described in a previous report[21] but no precise correlation with any biological or immunological component was identified. In contrast to hFIX, expression of β-galactosidase was transient in all immunocompetent mice injected with AdLacZ (n = 25). *LacZ* expression was detected in livers and lungs only during the first 3 weeks after virus inoculation (Figure 2c, d, g and h).

Persistence of hFIX and lacZ expression in immunodeficient mice

AdhFIX or AdLacZ viruses (5×10^9 p.f.u.) were similarly injected into the tail vein of mice deficient in either T cell function (C57BL/6-nu/nu mice), T and B cell function (SCID mice) or T, B, natural killer (NK) and lymphokine-activated killer (LAK) cell function (NIH-bg-nu-xid mice) in order to determine the role of the immune system in the persistence of transgene expression (Figure 1b, and Figure 2a, b, e and f). In all mice (n = 18), serum hFIX concentration increased rapidly to reach a peak value 2 days after treatment, and then remained relatively stable during the course of the study. Similar to C57Bl/6 mice, a slight progressive decrease of hFIX levels was none the less observed in all immunodeficient animals suggesting that nonimmunological factors (eg natural cell turn-over) are involved in this slow decline of transgene expression. This contrasts with the situation with immunocompetent

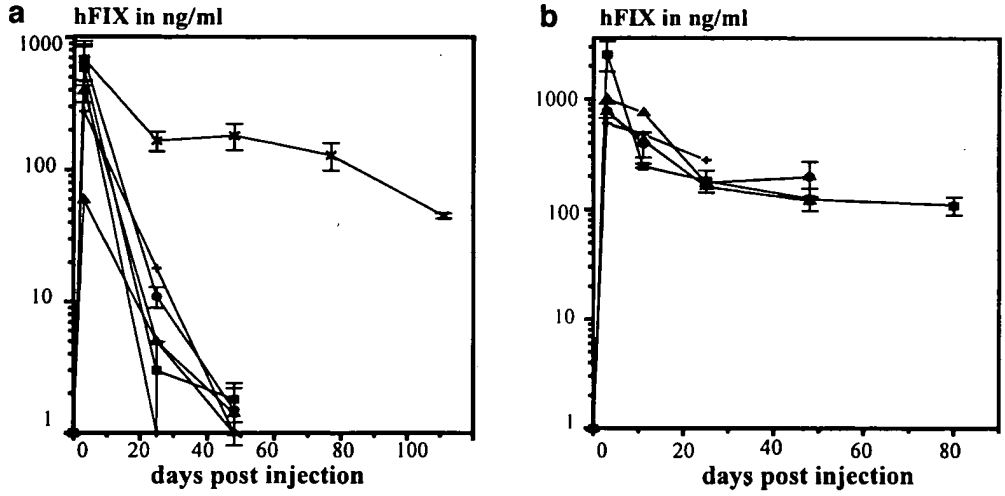

Figure 1 Persistence of hFIX expression in different strains of immunocompetent (a) and immunodeficient (b) mice injected intravenously with 5×10^9 p.f.u. of the AdhFIX vector. Blood samples were periodically collected and were tested by ELISA for hFIX concentrations. (a) Immunocompetent mice: C3H (●), mean of seven animals), BALB/c (■, mean of six animals), C57Bl/6 (x, mean of six animals at days 3, 11 and 77; mean of four animals at day 111), CBA/J (▲, two individual animals), CD1 (+, two individual animals). (b) Immunodeficient mice: NIH-bg-nu-xid (●, mean of six animals), SCID (■, mean of eight animals, except at day 80 (two animals)), BALB/c-nu/nu (▲, two individual animals), and C56Bl/6-nu/nu (+, two individual animals).

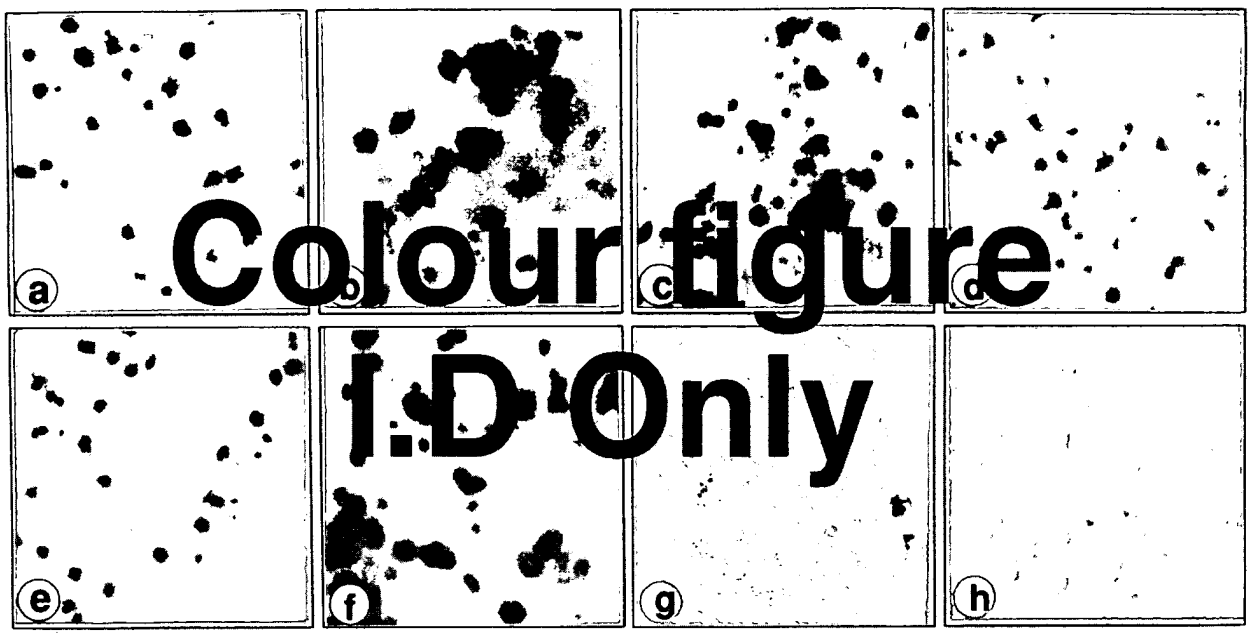

Figure 2 Persistence of β-galactosidase expression in immunocompetent and immunodeficient mice injected intravenously with 5×10^9 p.f.u. of the AdLacZ vector. The following panels are representative of the general pattern of β-galactosidase expression observed in all tested livers and lungs harvested from either immunodeficient or immunocompetent animals. Lungs (a and e) and livers (b and f) from SCID mice, livers from BALB/c mice (c and g) and lungs from C57Bl/6 mice (d and h) were recovered 10 days (a–d) or 60 days (e–h) after virus inoculation and were tested by X-gal staining for β-galactosidase expression.

mice (except C57Bl/6) in which hFIX levels diminish precipitously (see above). Similarly to results with hFIX, and in agreement with previous reports,[29–33] *lacZ* expression in liver, lung and heart recovered from mice injected with AdLacZ was stable in all immunodeficient animals (n = 16): 25% (mean ± 5%) of the liver cells stained positive for *lacZ* expression from day 4 to day 60 after intravenous injection of AdLacZ. In contrast, no *lacZ*-positive cells were found in immunocompetent mice (n = 25) 60 days after virus inoculation (Figure 2). These observations suggest that a host-specific immune response is involved in the rapid disappearance of hFIX and *lacZ* expression in these animals. The mechanisms responsible for the transient expression of *lacZ* and hFIX in immunocompetent mice are however probably not the same since hFIX expression is persistent in C57Bl/6 but not in CD1, BALB/c, CBA/J and C3H mice, while *lacZ* expression is transient in all tested immunocompetent animals.

Loss of hFIX detection correlates with the induction of anti-hFIX antibodies

In order to determine the immunological factor(s) responsible for the decline in hFIX expression, serum samples were recovered from C57Bl/6, BALB/c, CBA/J, C3H, CD1, nu/nu and NIH-bg-nu-xid mice injected with AdhFIX and were tested by ELISA for the presence of antibodies directed against either adenovirus (Figure 3) or the human transgene (Figure 4). Analysis of these sera revealed a strong correlation between hFIX expression and induction of anti-hFIX antibodies. While anti-adenovirus neutralizing antibodies were detected in the sera recovered from all immunocompetent mice (Figure 3), high levels of anti-hFIX antibodies were found only in BALB/c, C3H, CBA/J and CD1 mice but not in C57Bl/6 animals (Figure 4). As expected, no anti-adenovirus or anti-hFIX antibodies were detected in the sera of immunodeficient mice. In parallel, lungs and livers were regularly recovered from C57Bl/6, BALB/c and NIH-bg-nu-xid mice (three to six animals per strain) injected with AdhFIX and total DNA was prepared and analyzed for vector DNA persistence (Figure 5a). No significant difference in the persistence of AdhFIX DNA in liver (Figure 5a) and lung (data not shown) was found between BALB/c and C57Bl/6 animals indicating that prolonged detection of hFIX in the serum of C57Bl/6 mice is not related to a better stability of vector DNA or a longer persistence of the transduced cells in this mouse strain. These results suggest that the rapid disappearance of hFIX detection in immunocompetent mice (except C57Bl/6) is a consequence of the induction of specific antibodies against the human protein.

Extinction of lacZ expression correlates with the disappearance of AdLacZ DNA

Although the pattern of hFIX and *lacZ* expression is apparently similar in all animals except C57Bl/6 mice, the mechanisms responsible for the absence of persistence of *lacZ* and hFIX expression in immunocompetent mice are however not identical. Analysis of viral DNA copy number in liver and lung recovered from BALB/c, C57Bl/6 and SCID mice (three to six animals per strain) at 3 and 45 days after AdLacZ inoculation revealed a striking difference between the two types of vectors (Figure 5). Despite a progressive decline, AdhFIX DNA copy number was still relatively high in all tested immunocompetent animals 48 days after virus injection (over one copy per cell) showing good *in vivo* stability of aden-

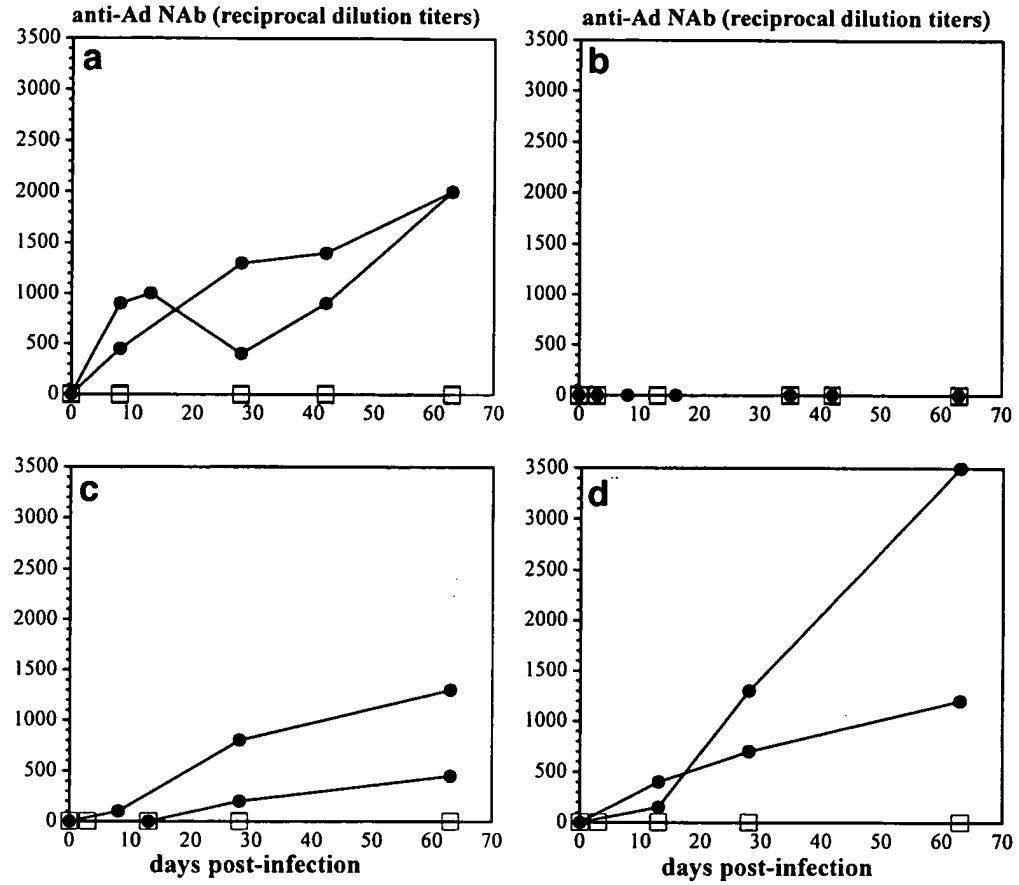

Figure 3 Induction of anti-adenovirus neutralizing antibodies in mice inoculated intravenously with AdhFIX. Blood samples were regularly collected and tested in an in vitro assay. Each line represents data from an individual animal. (a) C57Bl/6 mice; (b) NIH-bg-nu-xid mice; (c) CBA/J mice; (d) CD1 mice. Filled circles represent animals treated with the recombinant vector; open squares represent the mock-infected mice.

ovirus DNA in quiescent murine cells (Figure 5a). In contrast to AdhFIX, AdLacZ sequences declined very rapidly in both liver and lung tissues (Figure 5b, and data not shown) to reach undetectable levels at 45 days after virus injection. Similarly to AdhFIX, AdLacZ DNA was however relatively stable in organs isolated from immunodeficient mice (Figure 5b). Altogether, these observations suggest that AdLacZ DNA is rapidly eliminated as a result of the host immune reaction. In contrast, persistence of AdhFIX DNA, but not persistence of hFIX detection, is less sensitive to immunological factors.

Adenovirus-specific cytotoxic T lymphocytes (CTLs) are not involved in the rapid elimination of the AdLacZ transduced cells

The *in vivo* persistence of AdhFIX genomic DNA was unexpected given previous reports showing the induction of adenovirus-specific CTLs in mice injected with vectors expressing the bacterial β-galactosidase or alkaline phosphatase genes.[29–33] These observations led to the suggestion that the virus-specific cellular immune response was responsible for the rapid destruction of the transduced cells. In order to determine more precisely the role of the anti-adenovirus cellular immunity, we tested whether immunocompetent mice inoculated with AdhFIX that display long-term persistence of the transduced cells (C57Bl/6, CBA/J and C3H mice) and prolonged detection of the transgene expression (C57Bl/6 mice) similarly developed a CTL response against the viral antigens. In agreement with Yang *et al*,[29–33] a strong virus-specific CTL response was detected in all (n = 6) tested animals (Figure 6a and c; data not shown), independently of the pattern of expression of the transgene. The development of a cellular immune response specifically directed against the adenovirus antigens was further confirmed by experiments showing that AdLacZ viral particles can specifically stimulate the proliferation of spleen cells recovered from mice inoculated by AdhFIX (Figure 6b and d). However, in contrast to previous reports,[29–33] these data indicate that the induction of a cytotoxic cellular immune reaction directed against adenovirus antigens does not directly lead to a rapid destruction of the transduced cells. Furthermore, these results also suggest that the rapid elimination of the AdLacZ-transduced cells (Figure 5b) is not related to the presence of anti-adenovirus CTLs, but most probably to a CTL response directed against the β-galactosidase, as we have previously reported.[40] We cannot however completely exclude that the apparent slow decline of AdhFIX DNA copy number observed in the immunocompetent mice is a long-term consequence of the anti-adenovirus CTL reaction (Figure 5a).

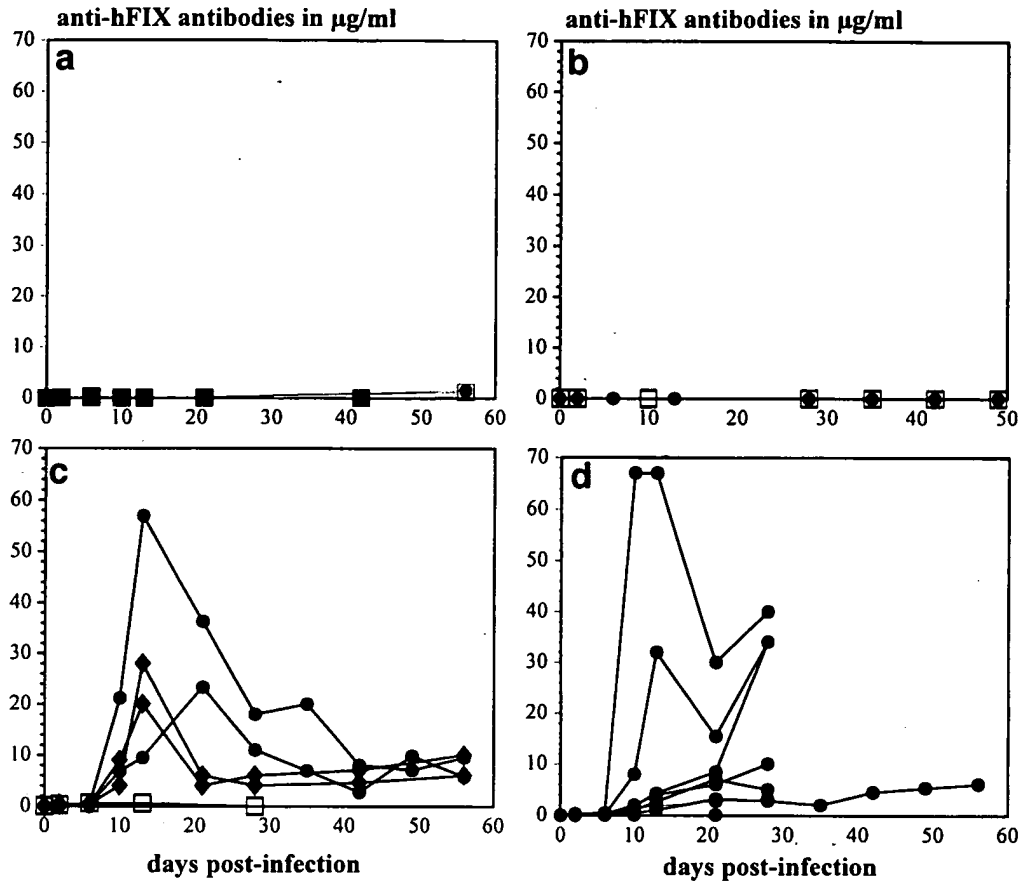

Figure 4 Induction of anti-hFIX antibodies in mice inoculated intravenously with AdhFIX. Periodic serum samples were collected and tested by ELISA for anti-hFIX antibody concentrations. Each line represents data from an individual animal. (a) C57Bl/6 mice (n = 5); (b) NIH-bg-nu-xid mice (n = 2); (c) CBA/J (●) and CD1 mice (◆); (d) C3H mice. Open squares represent the mock-infected mice.

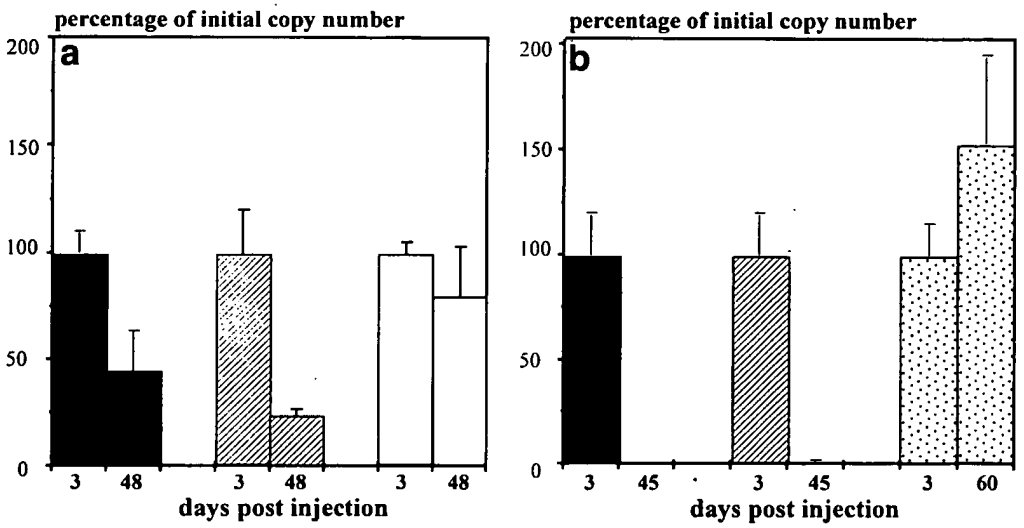

Figure 5 Persistence of AdhFIX and AdLacZ DNA sequences in hepatocytes of immunocompetent and immunodeficient mice. 5 × 10⁹ p.f.u. of AdhFIX (a) or AdLacZ (b) vectors were injected in the tail vein of BALB/c (filled bars, three mice), C57BL/6 (cross-hatched bars, six mice), NIH-bg-nu-xid (empty bars, three mice) and SCID (dotted bars, three mice) mice on day 0. Livers were recovered 3 and 45/48/60 days after virus inoculation. Total cellular genomic DNA was prepared and persistence of the viral DNA was quantified by Southern blot analysis and densitometry scanning of the autoradiographs. Persistence of adenoviral genomes is defined as the percentage of DNA copy number measured at 3 days after virus injection.

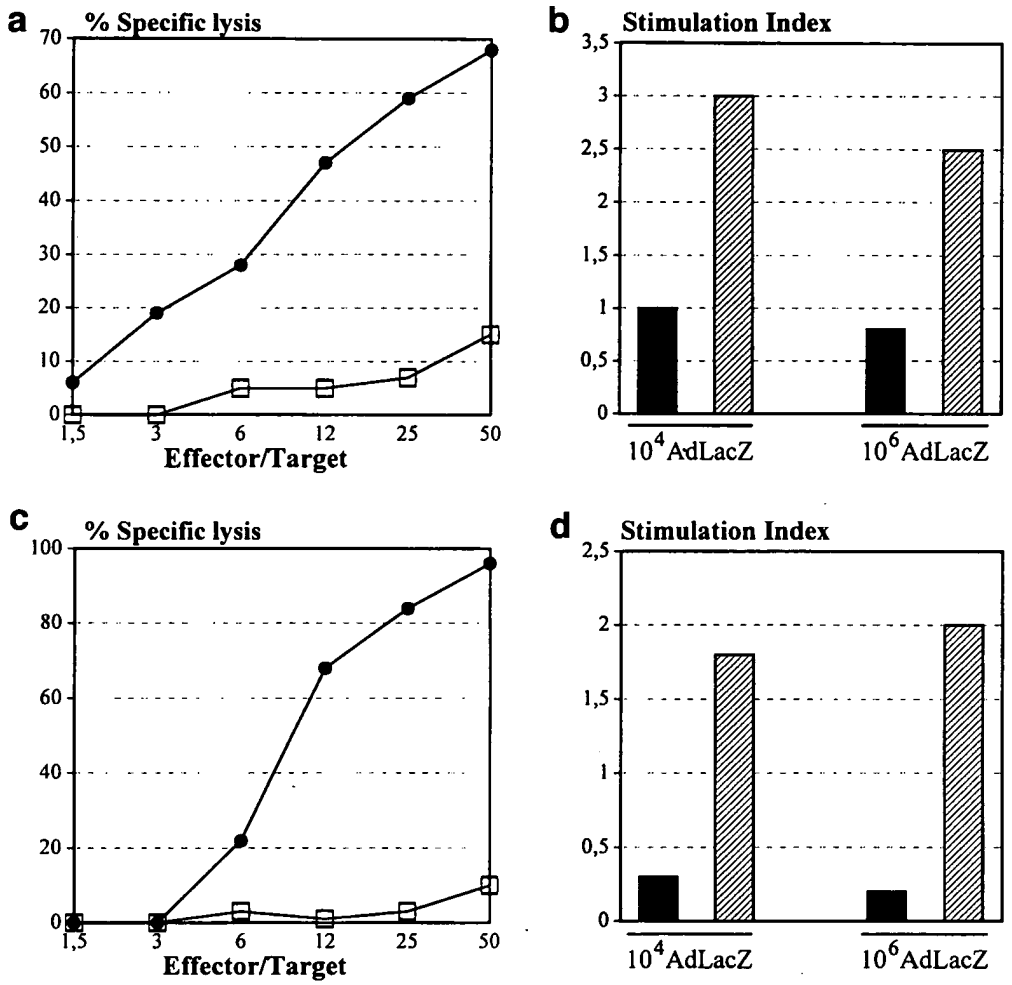

Figure 6 Induction of cellular immune response to adenovirus antigens in mice injected with AdhFIX. Four days after the last virus administration, splenocytes were harvested from C3H (a and b) and C57Bl/6 (c and d) mice inoculated intraperitoneally at days 0 and 10 with 5×10^8 p.f.u. of AdhFIX (circles) or PBS (squares). Splenocytes were restimulated in vitro for 6 days with either L929 (a) or EL-4 (c) cells infected with AdLacZ at a multiplicity of infection (MOI) of 200, and were tested for specific lysis on AdLacZ-infected L929 or EL-4 target cells, respectively. In parallel, splenocytes from mock-infected (filled bars) or AdhFIX-infected (cross-hatched bars) C3H (b) and C57Bl/6 (d) mice were analyzed for their T cell proliferative response to 10^4 and 10^6 AdLacZ adenovirus particles coated on the culture plates.

Discussion

The prospect of using E1-deleted adenovirus vectors to transfer potential therapeutic genes into a variety of quiescent and dividing cell types in the absence of residual expression of viral genes stimulated a broad interest in the possible treatment of a number of genetic diseases.[14–25] Recent data from *in vivo* studies in various animal models have however revealed that in most cases, only transient expression of the transgenes was obtained.[14–16,18,21] Despite the deletion of E1, current vectors still express viral genes encoding early (DNA binding protein) and late proteins (penton, hexon, fiber) at low levels (Refs 26–33; our unpublished data), suggesting that such proteins might be recognized as foreign by the immune system of the treated subjects, leading to immunological destruction of the transduced cells. Recent reports showing prolonged expression in animals treated with adenovirus vectors defective in both E1 and E2A and expressing no, or only low amounts, of viral proteins confirmed this initial hypothesis.[27,28,32] Novel vectors simultaneously deleted in E1 and E4 have therefore been recently generated in order to abolish the expression of viral antigens.[36–38]

In order to establish the optimal parameters required for a comparative *in vivo* evaluation of first generation E1-deleted adenovirus vectors (E1°) and second generation (E1°/E2A°, E1°/E4°, E1°/E2A°/E4°) vectors, we tested the influence of the mouse strain and the nature of the transgene on the persistence of expression in mice treated with recombinant E1-deleted vectors. Experiments were performed using nine differents strains of immunocompetent and immunodeficient mice and two E1-deleted adenovirus vectors expressing the human coagulation factor IX or the bacterial β-galactosidase genes.

As previously reported for human and canine FIX and human α-AT,[15,21,39] we confirm that the animal strain significantly influences the apparent persistence of hFIX expression. We extend these observations with the identification of the immunological factor responsible for the transient persistence of hFIX in the immunocompetent

mice tested in this study, with the exception of C57Bl/6. All immunocompetent animals, except C57Bl/6 mice, injected intravenously with the AdhFIX vector were found to develop high levels of anti-hFIX antibodies within 15 days of treatment. These levels, ranging from 3 μg/ml to 64 μg/ml, are significantly higher than the background levels (<0.3 μg/ml) observed in mock-injected mice and were found to be inversely correlated with the persistence of hFIX in the serum of the animals. Therefore, induction of anti-hFIX antibodies at levels that are above a threshold of about 1 μg/ml seems to be associated with a loss of detection of hFIX in the serum. As expected, none of the immunodeficient mice developed anti-hFIX antibodies and, similarly to the C57Bl/6 mice, presented a stable pattern of hFIX expression. Absence of production of anti-hFIX antibodies in C57Bl/6 animals was not related to an inability of the mice to develop a humoral response since all treated C57Bl/6 animals produced high levels of neutralizing antibodies directed against the adenovirus particles. The absence of detection of hFIX in mice that develop anti-hFIX antibodies could be explained by at least two hypotheses: (1) anti-hFIX antibodies may participate in the formation of immune complexes with the secreted hFIX. The presence of such complexes may block the detection of the hFIX by ELISA, or may facilitate the elimination of the circulating hFIX molecules; (2) these antibodies may trigger an antibody-dependent cell cytotoxicity[41] (ADCC) leading to destruction of the transgene-expressing cells. Analysis of the AdhFIX DNA copy number in various tissues recovered from the injected mice does not support the latter hypothesis: viral DNA was always relatively stable during the course of our study, indicating that the transduced hepatocytes or pulmonary cells were not rapidly eliminated in any of the treated mice. The first hypothesis is further supported by the detection over 2 months of hFIX-specific RNAs in organs recovered from the immunocompetent mice (data not shown).

Interestingly, all tested immunocompetent mice injected with AdhFIX developed a cytotoxic cellular immune response directed specifically against the adenovirus antigens. However, these CTLs were not sufficient to induce a rapid elimination of the transduced liver and lung cells, as demonstrated by the relative stability of the AdhFIX DNA copy number in these tissues. This observation contradicts results published by Yang *et al*[29–33] indicating that cellular immunity to viral antigens constitutes the dominant factor responsible for the rapid elimination of the transduced cells and hence for extinction of transgene expression. The reason for this discrepancy remains unclear, but we cannot exclude the possibility that the accumulation of high concentrations of foreign immunogenic proteins (eg β-galactosidase) activates the immune system and stimulates the development of a stronger specific immune reaction directed against the virus antigens. It is also possible that the inflammatory reaction often associated with the use of first generation adenovirus vectors might potentiate the immune response directed against the transgene-encoded products. Such synergistic effects remain however to be experimentally demonstrated.

Compared with immunocompetent mice, an apparent better stability of AdhFIX DNA was nonetheless observed in the organs of the immunodeficient animals similarly injected with AdhFIX: 25 and 45% of the initial DNA copy number were detected 48 days after virus injection in the liver of BALB/c and C57Bl/6 mice, respectively, while 80% of the initial DNA copy number was found in the liver of NIH-bg-nu-xid mice. Whether this progressive diminution of the AdFIX DNA is the consequence of the induction of CTLs directed against the adenovirus antigens remains to be determined. The weak (or absence of) effect of the anti-adenovirus CTLs on the persistence of the transduced cells also indicates that the rapid loss of the AdLacZ sequences that occurred between 3 and 45 days in the hepatocytes and pulmonary cells of BALB/c and C57Bl/6 mice, but not in immunodeficient mice, reflects the induction of a specific immune response directed against the *lacZ* transgene. We recently demonstrated that administration of AdLacZ to BALB/c mice could induce a strong specific cellular and humoral response directed against the β-galactosidase protein.[40] The specific anti-β-galactosidase CTLs are most probably responsible for the rapid decline in AdLacZ DNA copy number in the tissues of the treated animals, through the destruction of *lacZ*-expressing cells.

Altogether our results show that: (1) persistence of transgene expression in adult immunocompetent mice injected with E1-deleted recombinant adenovirus vectors is much better than previously suggested,[15,26–33] provided that the animals are tolerant to the protein encoded by the transgene (eg hFIX in C57Bl/6 mice). This observation confirms recent data generated with an E1-deleted adenovirus encoding the murine erythropoietin gene;[35] (2) the mouse strain selected for the study does influence the pattern of detection or expression of the transgene, depending on the ability of the animal to develop an immune response against the transgene product. Moreover, the type of immune response elicited in the treated animals (humoral *versus* cellular) also significantly influences the persistence of the transduced cells: loss of *lacZ* expression is correlated with an elimination of the transduced cells, while loss of hFIX detection is not; (3) consequently, the nature of the selected transgene might significantly alter the conclusions of the study regarding the respective roles of the adenovirus-specific and transgene-specific immunological factors in the persistence of transgene expression; (4) although the administration of recombinant adenoviruses induces a detectable cellular immunity to the viral antigens, the transduced cells persist during a relatively long period of time (over 2 months; this work and data not shown). This observation suggests that the use in previous studies of adenoviruses encoding transgenes of various nonmurine origins might have led to an overestimation of the role of anti-adenovirus CTLs in the transient expression of the transgenes in mice. This study also indicates that any future comparison of the recently developed novel generations of adenovirus vectors should be done with vectors that do not express foreign genes or with animal models tolerant to the products encoded by the transgenes.

Materials and methods

Animal studies

Immunocompetent and immunodeficient female mice were purchased at 4 weeks of age from Iffa Credo (l'Arbresle, France) or the Charles River Breeding Facility (Saint-Aubin-les-Elbeufs, France). All immunocompetent

(C57Bl/6, H-2^b; BALB/c, H-2^d; CBA/J, H-2^k; C3H, H2^k; CD1, outbred) and immunodeficient[42–44] (BALB/c-nu/nu and C57Bl/6-nu/nu; C.B17-scid/scid; NIH-bg-nu-xid) animals were bred in a specific pathogen-free environment. 5×10^9 p.f.u. of recombinant adenovirus diluted in a solution of 10 mM Tris, 1 mM $MgCl_2$, 10% glycerol were injected in the tail vein of each mouse in a volume of 150 µl. Blood samples were collected at different times after injection and the corresponding sera were stored at –20°C.

Recombinant adenovirus vectors

The E1/E3-deleted AdhFIX vector was constructed by homologous recombination in 293 cells[11] between a plasmid DNA fragment containing the hFIX cDNA[45] under the control of the mouse phosphoglycerate kinase promoter[46] (PGKpro), and the genomic *Cla*I DNA fragment isolated from the H5dl324 virus.[22] The recombinant virus was plaque-purified and amplified on 293 cells, and virus genome integrity was analyzed by restriction enzyme digestion and Southern blot analysis.[47,48] The E1/E3-deleted AdLacZ vector used in this study has been previously described.[22]

Determination of human factor IX (hFIX) concentration

Concentration of human factor IX protein in mouse sera was determined using a commercial enzyme-linked immunosorbent assay (ELISA) (Asserachrom IX: Ag kit; Diagnostica Stago, Asnières, France).

Anti-adenovirus antibody assay

Concentrations of adenovirus-specific antibodies were determined by ELISA. Briefly, 96-well plates were coated overnight at 4°C with 50 µl of buffer (200 mM $NaHCO_3$, 81 mM Na_2CO_3, pH 9.5) containing 1.6×10^7 p.f.u. of AdLacZ. Wells were then successively washed five times with PBS, saturated with 200 µl of 2% bovine serum albumin (BSA) in PBS, rinsed once in PBS and incubated with diluted serum (1:10 000) for 90 min at room temperature. Then 100 µl per well of a biotin-SP-conjugated goat anti-mouse IgG + IgM (H+L) antibody (dilution 1:200; Jackson Immunoresearch Laboratories, France), and 100 µl per well of a streptavidine biotinylated horseradish peroxidase complex (dilution 1:500; Amersham, Les Ullis, France) were each successively incubated for 1 h at room temperature. Finally, 100 µl per well of the substrate solution (5 mg *O*-phenylenediamine dihydrochloride, 5 µl H_2O_2, 12.5 ml 0.2 M Na_2HPO_4, 0.1 M citric acid) were added to each well and substrate conversion was stopped by the addition of 30 µl per well of 3 M H_2SO_4. Absorbance was measured at 490 nm.

Neutralizing anti-adenovirus antibody assay

Determination of anti-adenovirus neutralizing antibodies was done as described previously[40] using decomplemented mouse sera.

Anti-hFIX antibody assay

Murine antibodies directed against hFIX were detected by ELISA: 96-well flat-bottomed MaxiSorp plates (Polylabo, Strasbourg, France) were coated overnight at 4°C with 600 ng of hFIX protein (Sigma ImmunoChemicals, Chesnes, France) diluted in a solution of 200 mM $NaHCO_3$, 81 mM Na_2CO_3, pH 9.5. Plates were then saturated with 250 µl of a 3% BSA in PBS for 1 h at 37°C and were successively incubated for 2.5 h at room temperature with 100 µl per well of diluted murine sera (1:50), 1 h with 100 µl per well of a biotin-SP-conjugated goat anti-mouse IgG + IgM (H+L) antibody (dilution 1:1000; Jackson Immunoresearch Laboratories) and 1 h with 100 µl per well of a streptavidin biotinylated horseradish peroxidase complex (dilution 1:500; Amersham). After each incubation, wells were washed six times with 0.1% Tween 20, 10 mM EDTA, PBS (250 µl per well). Finally, 100 µl per well of the substrate solution (5 mg *O*-phenylenediamine dihydrochloride, 5 µl H_2O_2, 12.5 ml 0.2 M Na_2HPO_4, 0.1 M citric acid) were incubated for 3 min at room temperature and 30 µl per well of 3 M H_2SO_4 were added to stop the enzymatic reaction. Absorbance was measured at 490 nm. Anti-hFIX antibody concentrations were determined using a characterized anti-hFIX monoclonal antibody (Sigma) as an internal standard.

Quantitative analysis of viral DNA in mouse tissues

Mice injected intravenously with 5×10^9 p.f.u. of AdhFIX or AdLacZ were killed 3 days, 2 weeks and 7 weeks after virus injection. Total cellular DNA was prepared from liver or lung by SDS-proteinase K cell lysis followed by phenol-dichloromethane extraction and ethanol precipitation. Ten micrograms of DNA were digested by *Bam*HI (AdhFIX) or *Eco*RV (AdLacZ) and analyzed by Southern blot[47] using appropriate ^{32}P dCTP-labelled DNA probes (the hFIX cDNA or the β-galactosidase gene). A densitometry scanning of the autoradiographs was performed with the model GS-700 Imaging Densitometer (BioRad, Ivry-sur-Seine, France); the data were analyzed using the Molecular Analyst/PC software (BioRad).

X-gal histochemistry

Tissues recovered from killed mice were fixed at 4°C in 2% formaldehyde, 2% glutaraldehyde for 1 h, rinsed in PBS and incubated for 24 h or 48 h at 30°C in 5 mM $K_3Fe(CN_6)$, 5 mM $K_4Fe(CN_6)$, 2 mM $MgCl_2$, 1 mg/ml 5-bromo-4.chloro.3-indolyl-β-D-galactopyranoside (X-gal). Tissues were then rinsed in PBS, post-fixed in 5% formamide/PBS for 24 h at 4°C, dehydrated in alcohol and embedded in paraffin. Random sections (5 µm) were examined for β-galactosidase expression. The percentage of *lacZ*-expressing cells was estimated by random microscopic observation of different fields in the organ sections, using a magnification of 20. Values are expressed as the mean value obtained for three to five mice per strain and per time-point.

Cytotoxic T lymphocyte (CTL) assay

Six-week-old female C57Bl/6 and C3H mice were immunized at day 0 and 10 with two intraperitoneal injections of 5×10^8 p.f.u. of AdhFIX in 100 µl saline. Negative control mice were mock-infected with PBS in parallel. All mice were killed at day 14 and splenocytes from two immunized animals were pooled and stimulated *in vitro* for 6 days with either EL-4 cells (H2^b; C56Bl/6 mice) or L929 (H2^k; C3H mice) cells infected with AdLacZ at an MOI of 200. The stimulation ratio was 50:1 (splenocytes:stimulator cells). Cytolytic activity was determined in a standard 4-h chromium release assay; 2×10^6 target EL-4 cells or L929 cells infected for 24 h with AdLacZ (MOI 200) were labelled with 100 µCi of ^{51}Cr (Amersham) for 1 h at 37°C. Effector splenocytes were plated at different effector-to-target ratios (50:1,

25:1, 12:1, 6:1, 3:1, 1.5:1) in 96-well plates containing 4×10^4 target cells per well. After incubation for 4 h at 37°C, plates were centrifuged and aliquots of 100 µl supernatant were collected and counted in a gamma counter. Spontaneous release was determined by culturing target cells in culture medium. Maximum release was determined by lysing target cells with 1 N HCl. Results of duplicate culture are expressed as: % specific Cr release = 100 × (experimental release – spontaneous release)/(maximun release – spontaneous release).

Lymphoproliferation assay

A fraction of the splenocytes recovered from the mice used for the CTL assay were analyzed for their proliferative response to AdLacZ adenovirus particles. Splenocytes were plated in 96-well plates at 2×10^5 cells per well and stimulated for 4 days at 37°C with UV-inactivated AdLacZ particles (0, 10^4 and 10^6 p.f.u.) previously coated on the culture plates. On day 4, splenocytes were pulsed overnight with ^{3}H-thymidine (1 µCi per well; Amersham) before harvesting. ^{3}H-thymidine incorporation was measured using a beta-counter. The stimulation index is calculated as the ratio between the values of ^{3}H-thymidine incorporation by the stimulated cells *versus* the unstimulated cells.

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